ZOOLOGICAL SCIENCE 11: 441–444 (1994)

Neuron-like morphology expressed by perinatal rat C-cells *in vitro*

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ABSTRACT—Thyroid C-cells (calcitonin-producing cells) are endocrine derivatives of the neural crest. The morphological plasticity of rat C-cells was examined under cell culture. In a primary culture of perinatal rat thyroid glands, a small number of C-cells were found to extrude neurite-like processes, some of which reached 350μm in length. The processes frequently branched and had varicosity-like structures. The processes were intensely stained with anti-α-tubulin antibody, suggesting that microtubular cytoskeleton participated in their elongation and maintenance. In primary cultures of C-cells derived from postnatal rats at day 2 or later, no neurite-like processes were observed. These findings suggest that at least some C-cells in the perinatal rat thyroid retain the potential to extrude neurite-like processes, as do chromaffin cells in adrenal medulla, another type of crest-derived endocrine cell.

INTRODUCTION

Although calcitonin-producing cells (C-cells) in the thyroid gland are classified as endocrine cells, they have phylogenetic, embryological and biochemical relationships with enteric serotonergic neurons [1]. As befits their neuroectodermal origin, C-cells have several neuronal properties. They produce calcitonin gene-related peptide (CGRP), a putative neurotransmitter, as an alternative product of the calcitonin gene [12]. C-cells are capable of synthesizing serotonin from L-tryptophan and accumulate it in their secretory granules with neuron-specific serotonin binding protein [1]. Our recent study [9] revealed that C-cells express neural cell adhesion molecules on their surfaces. Neuronal characteristics of C-cells have also been confirmed by electrophysiological techniques [5, 13, 14]. Although C-cells share some neuronal properties, they do not display neuron-like morphological features in the thyroid gland [4].

In this communication, we report that a small number of C-cells derived from perinatal rats displayed neuronal morphology *in vitro*, suggesting their morphological plasticity.

MATERIALS AND METHODS

For immunohistochemical study, thyroid glands were excised from 20-day-old fetuses or 9-week-old male rats of Wistar strain. The day on which sperm were observed in vaginal smears was designated as day 0 of pregnancy. The glands were fixed in freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. After being rinsed with phosphate-buffered saline (PBS), the specimens were immersed in 20% sucrose in PBS at 4°C for 24 hr, and subsequently frozen in an OCT compound (Miles). Transverse sections of 6μm in thickness were cut on a cryostat and mounted on glass slides coated with egg white.

Calcitonin immunoreactivity in the sections was detected using rabbit anti-human calcitonin antiserum (1 : 800, ICN ImmunoBiologicals) and rhodamin-labeled goat anti-rabbit IgG antiserum (1 : 100, Cappel). The specimens were observed under a NIKON DIAPHOT-TMD microscope equipped with fluorescence optics. The procedures were detailed in our previous paper [8].

For primary cultures, the thyroid glands were dissected out from perinatal rats ranging in age from embryonic day 16 (E16) to postnatal day 4 (P4), and dispersed with collagenase (Worthington, CLS II) and Dispase (Godo Shusei) [7]. The dispersed cells were cultured on glass coverslips (14 mm in diameter) in 24-well multidishes (Falcon) with Dulbecco's modified Eagle's medium (GIBCO) containing 5% fetal bovine serum (GIBCO). The cell density was controlled to obtain approximately 500 C-cells/well after 48 hr of incubation, as detailed in our previous paper [7]. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Primary cultures were also prepared from the thyroid glands of young (4- and 9-week-old) and aged (80-week-old) rats as described above.

After being incubated for 48hr, the cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 1 hr, and permeabilized with 0.25% Triton X-100 for 10 min. Calcitonin was immunocytochemically detected as described above, and the number of neuron-like C-cells, which were defined as calcitonin-immunoreactive cells with at least one process longer than 150μm, were counted. In some experiments, CGRP was detected using rabbit anti-rat CGRP antiserum (1 : 400, Peninsula). For double-immunostaining of calcitonin and α-tubulin, mouse monoclonal anti-α-tubulin antibody (1 : 500, BioMakor) and fluorescein-labeled goat anti-mouse IgG antiserum (1 : 100, Kirkegaard and Perry Lab) were used in combination with the immunostaining of calcitonin described above. The specificity of the immunoreactions was determined by omitting the primary antibody, or by preincubation of the antibody with an excess of antigen. No specific immunoreactivity was found in these controls.

RESULTS AND DISCUSSION

In the sections of thyroid gland, C-cells were oval or

Accepted June 6, 1994
Received March 28, 1994

polygonal in shape, and were located close to the basal portion of the follicular epithelium (Fig. 1). In the E20 fetal thyroid, some of the C-cells were found to extrude short processes (Fig. 1A, *arrows*). The length of these processes was estimated to be 40μm at most. In contrast, no processes were observed in adult thyroid C-cells (Fig. 1B).

To determine whether these C-cells extend processes *in vitro*, the thyroid glands of both fetal and adult rats were dissociated into single cells and grown in culture. In the cultures of 9-week-old rat thyroid, C-cells displayed oval or triangular shapes. Although a few C-cells had short processes of up to 30μm, C-cells with long processes were not observed in the cultures of adult thyroid up to day 5 *in vitro* (data not shown). In the cultures of E20 fetal thyroid glands, most of the C-cells exhibited ovoid or triangular shapes, as in the cultures of adult thyroid (Fig. 2A). However, a small fraction of these C-cells displayed neuron-like features (Fig. 2 B-D). Most of the neuron-like C-cells were monopolar, and the processes frequently bifurcated. Varicosity-like structures were observed along some of these processes (*arrows* in Fig. 2 B and C). The cytoplasm, including the processes, of the C-cells was filled with calcitonin and CGRP immunoreactant. All the C-cells examined expressed CGRP immunoreactivity irrespective of whether the C-cells possessed the neurite-like processes. The longest process reached 350μm in length. Two to seven neuron-like C-cells (0.4–1.4%) were detected among approximately 500 C-cells in each culture well. Prolonged incubation of up to 5 days did not increase the proportion of neuron-like C-cells (data not shown).

The neurite-like processes of C-cells were intensely stained with anti-α-tubulin antibody (Fig. 2 E and F), suggesting that microtubular cytoskeleton participated in the formation and/or maintenance of these processes. Microtubules in neurites are stabilized by microtubule-associated proteins, such as MAP-2 and tau [15]. Our recent study revealed that both thyroid C-cells and a C-cell line produce tau-immunoreactive protein with an apparent molecular mass of 110,000 (Nishiyama *et al.*, in preparation), probably corresponding to the high molecular weight tau found in PC-12 cells [3] and neuroblastoma cells [2]. Therefore, it is plausible that microtubules in the processes of the neuron-like C-cells are associated with the high molecular weight tau protein.

The results of this study indicated that a small percentage of fetal rat C-cells, but none of adult origin, had the ability to extend long neurite-like processes. Next, we determined the ratio of neuron-like C-cells in thyroid cell cultures of perinatal rats of varying ages. As shown in Table 1, neuron-like C-cells were also observed at almost the same ratio in cultures of E19 fetal rat thyroid glands as in those of E20 fetuses, and at much lower ratios in cultures of E17, E18, P0 and P1 rat thyroid glands. No neuron-like C-cells were detected in the thyroid cell cultures of E16 fetuses, P2 and P3 pups (Table 1), or P28, P63 and P560 rats (data not shown). These results show that neuron-like C-cells appeared in thyroid cell cultures during only a limited perinatal period.

A question arises as to why only a subpopulation of C-cells extend processes in thyroid cultures of perinatal rats. This implies a heterogeneity in the microenvironment surrounding the C-cells or in the properties of C-cells themselves. To answer this question, it is important to know whether C-cells without process have the potential to extend processes in response to some factor(s). An attempt to induce neurite-like process outgrowth in the C-cells *in vitro* is now in progress using various growth factors and hormones.

Although the neural crest origin of C-cells has been confirmed by several investigators [6, 10, 11], the developmental process of restricting C-cell phenotypic traits in the neural crest lineage remains to be elucidated. Our findings suggested that a small number of C-cells from perinatal rats, especially those obtained one or two days before birth, had the potential to extrude *in vitro* long neurite-like processes, a hallmark of the neuronal phenotype. Future experiments using this culture system will shed light upon commitment and

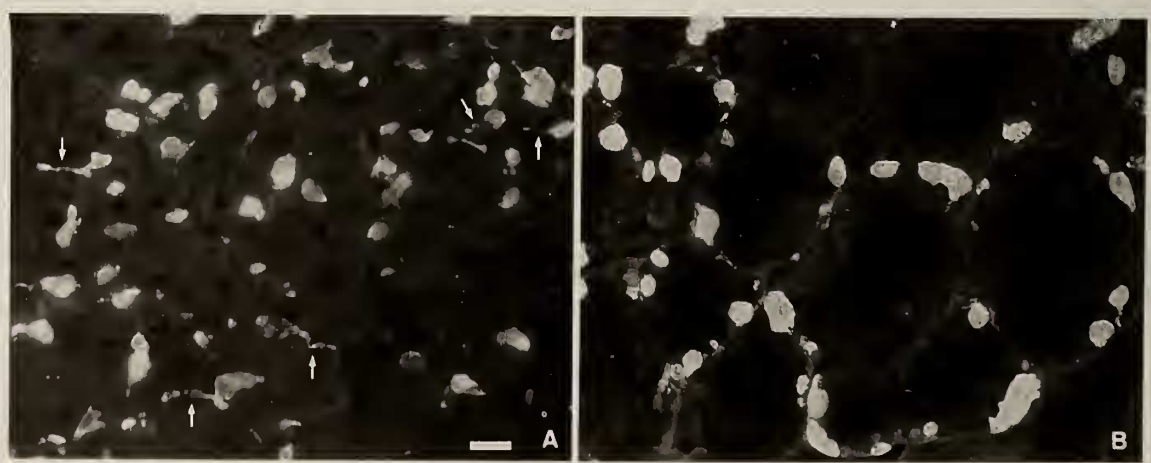

Fig. 1. Morphology of C-cells in the thyroid glands of fetal (A) and young adult (B) rats. Transverse sections of 20-day-old rat fetuses (A) and 9-week-old rats (B) were immunostained using an anti-calcitonin antiserum. Fetal C-cells appeared to extrude short processes (*arrows* in A). Scale bar=20μm.

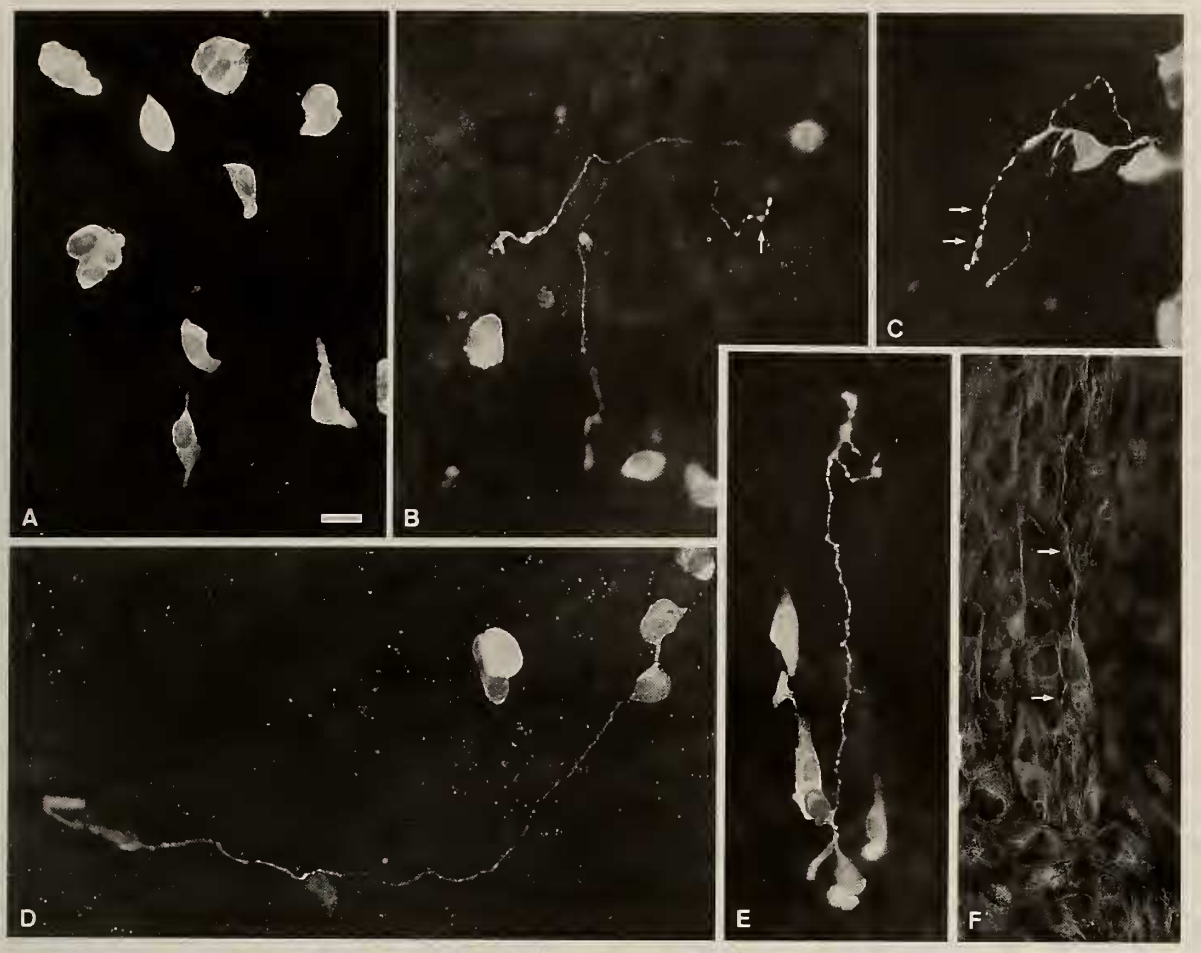

FIG. 2. C-cells in primary culture of dissociated thyroid glands of 20-day-old rat fetuses. The cells were cultured for 48 hr, and immunostained with antisera to calcitonin (A-C) and calcitonin gene-related peptide (D). C-cells with neurite-like processes were observed in the cultures at a low ratio (B-D). Their processes frequently branched and had varicosity-like structures (*arrows* in B and C). Calcitonin (E) and α-tubulin (F) immunoreactivities were detected in the same specimen. The neurite-like process of the C-cell was intensely reactive with anti-α-tubulin antibody (*arrows* in F). Scale bar=20μm.

TABLE 1. Frequency of neuron-like C-cells in primary cultures derived from rat thyroid glands of various ages

Age		Number of Neuron-like C-cells per 1,000 C-cells
Embryonic day	16	0
	17	0.1±0.07
	18	1.2±0.73
	19	7.3±1.54
	20	8.4±2.16
Postnatal day	0	2.2±1.35
	1	0.2±0.09
	2	0
	3	0

Thyroid glands of rat fetuses or pups at the ages indicated were enzymatically dissociated and cultured for 48 hr. The cells were immunostained using an anti-calcitonin antiserum, and the ratios of neuron-like C-cells, which were defined as calcitonin-immunoreactive cells bearing at least one process longer than 150μm, were determind. At least three samples, each of which contained approximately 500 C-cells, were counted for each experiment. The values are means±SEM of four independent experiments.

the plasticity of the phenotype in C-cells.

REFERENCES

1 Bernd P, Gershon MD, Nunez EA, Tamir H (1979) Localization of a highly specific neuronal protein, serotonin binding protein, in thyroid parafollicular cells. Anat Rec 193: 257–268

2 Couchie D, Mavilia C, Georgieff IS, Liem RKH, Shelanski ML, Nunez J (1992) Primary structure of high molecular weight tau present in the peripheral nervous system. Proc Natl Acad Sci USA 89: 4378–4381

3 Goedert M, Spillantini MG, Crowther RA (1992) Cloning of a

big tau microtubule-associated protein characteristic of the peripheral nervous system. Proc Natl Acad Sci USA 89: 1983–1987

4 Kameda Y (1987) Localization of immunoreactive calcitonin gene-related peptide in thyroid C cells from various mammalian species. Anat Rec 219: 204–212

5 Kawa K (1988) Voltage-gated sodium and potassium currents and their variation in calcitonin-secreting cells of the chick. J Physiol 399: 93–113

6 Le Douarin N, Fontaine J, Le Lièvre C (1974) New studies on the neural crest origin of the avian ultimobranchial glandular cells—Interspecific combinations and cytochemical characterization of C cells based on the uptake of biogenic amine precursors. Histochemistry 38: 297–305

7 Nishiyama I, Fujii T (1989) Somatostatin-immunoreactive C-cells in primary culture of fetal, neonatal and young rat thyroid glands. Biomed Res 10: 353–359

8 Nishiyama I, Fujii T (1992) Laminin-induced process outgrowth from isolated fetal rat C-cells. Exp Cell Res 198: 214–220

9 Nishiyama I, Seki T, Oota T, Ohta M, Ogiso M (1993) Expression of highly polysialylated neural cell adhesion molecule in calcitonin-producing cells. Neuroscience 56: 777–786

10 Pearse AGE, Polak JM (1971) Cytochemical evidence for the neural crest origin of mammalian ultimobranchial C cells. Histochemie 27: 96–102

11 Polak JM, Pearse AGE, Le Lièvre C, Fontaine J, Le Douarin NM (1974) Immunocytochemical confirmation of the neural crest origin of avian calcitonin-producing cells. Histochemistry 40: 209–214

12 Sabate MI, Stolarsky LS, Polak JM, Bloom SR, Varndell IM, Ghatei MA, Evans RM, Rosenfeld MG (1985) Regulation of neuroendocrine gene expression by alternative RNA processing. J Biol Chem 260: 2589–2592

13 Sand O, Jonsson L, Nielsen M, Holm R, Gautvik KM (1986) Electrophysiological properties of calcitonin-secreting cells derived from human medullary thyroid carcinoma. Acta Physiol Scand 126: 173–179

14 Sand O, Ozawa S, Gautvik KM (1981) Sodium and calcium action potentials in cells derived from a rat medullary thyroid carcinoma. Acta Physiol Scand 112: 287–291

15 Tucker RP (1990) The roles of microtubule-associated proteins in brain morphogenesis: a review. Brain Res Rev 15: 101–120

ZOOLOGICAL SCIENCE 11: 445–449 (1994)

Proliferation of Pituitary Cells in Streptozotocin-induced Diabetic Mice: Effect of Insulin and Estrogen

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ABSTRACT—Insulin has growth-stimulatory actions in various tissues. The present study is aimed to clarify whether insulin stimulates the proliferation of anterior pituitary cells. Estrogen is the mitogenic factor in pituitaries and female reproductive tracts. We studied the insulin-estrogen relationship in the pituitary cell proliferation. The proliferation of uterine epithelial cells was also studied, since the uterus was one of the most typical estrogen-responsive organs. Ovariectomized ICR mice were given streptozotocin (STZ, 100 mg/kg) intraperitoneally to make mice insulin-deficient. Estradiol-17β (50 μg, E_2) and insulin (0.2 or 0.4 IU per twice a day) were given in STZ-treated mice. Insulin significantly stimulated the mitosis of pituitary cells and PRL cells in both normal control mice and STZ-treated diabetic mice. E_2 stimulated the mitosis of pituitary cells (10.93 ± 0.73 cells/mm^2), compared with controls (3.59 ± 0.67 cells/mm^2) in control mice. In STZ-treated mice E_2 failed to increase the mitosis (4.91 ± 0.99 cells/mm^2), compared with controls (2.47 ± 0.43 cells/mm^2). Insulin recovered the diminished response to estrogen in pituitary cells of STZ-treated mice to the level comparable to control mice. In the uterus insulin at the high dose used stimulated the proliferation of luminal epithelial cells in normal control mice. However, insulin deficiency did not alter the responsiveness of uterine epithelial cells to estrogen. The present study suggests that insulin is involved in the proliferation of pituitary cells and probably uterine luminal epithelial cells, but the mechanism of insulin action on the cell proliferation may differ between pituitary cells and uterine cells.

INTRODUCTION

Estrogen stimulates proliferation of various cells including pituitary cells and uterine cells. Recent reports concerning the estrogen-induced cell proliferation suggest that estrogen action is not a direct action and is mediated by autocrine or paracrine growth factors [24, 27, 32]. Transforming growth factor α and epidermal growth factor are growth factors for pituitary cells, particularly for prolactin (PRL) cells [3]. It is highly probable that other growth factors are involved in pituitary cell proliferation. Insulin is known to have growth-promoting activity in various tissues [26]. Insulin is indispensable for estrogen-induced pituitary growth [6]. Pituitary tumor cells, GH_3 cells, require insulin for the optimal growth in serum-free medium [11]. The present study was undertaken to clarify effects of insulin on the estrogen-induced proliferation of pituitary cells in ovariectomized mice made diabetic by streptozotocin (STZ) administration. The proliferation of PRL cells was particularly examined, since insulin stimulates PRL secretion [13, 25]. Uterine growth is regulated by estrogens and progesterone. The proliferation of uterine luminal epithelial cells in STZ-treated diabetic mice was also studied in the present study.

MATERIALS AND METHODS

Adult female mice (2 month old) of the Jcl:ICR strain (Clea Japan) were used. They were kept under temperature-controlled conditions, and given food and water *ad libitum*. All mice were ovariectomized under ether anesthesia. Ten days after ovariectomy, STZ (Wako Pure Chemicals) was intraperitoneally given at a dose of 100 mg/kg BW. STZ was dissolved in 0.05 M citric acid (65 mg/ml). Control mice were given the vehicle. All mice had been in advance deprived of food for 16 hr before STZ treatment.

Estrogen treatment

Estradiol-17β (E_2, Sigma), dissolved in sesame oil (1 mg/ml), was subcutaneously given 7 days after STZ treatment at a dose of 50 μg. Control mice were given sesame oil.

Insulin treatment in STZ-treated mice

Seven days after STZ treatment insulin (0.2 or 0.4 IU for each mouse, Novo Actrapid MC, Novo Nordisk), diluted in saline (1 or 2 IU/ml), was intraperitoneally given twice a day (9.00 AM and 5.00 PM) for 3 days. Control mice were given the vehicle. On the last day insulin was given only in the morning, and 5 hr later pituitaries were collected for the study.

The mitotic activity of pituitary cells and uterine epithelial cells

Colchicine (Wako Pure Chemicals), dissolved in saline (1 mg/ml), was subcutaneously given at a dose of 5 mg/kg BW. The pituitary gland and uterine horns were removed 5 hr after the colchicine injection, that is, 48 hr after estrogen treatment, and then fixed in Bouin's solution. The pituitary glands and uteri were embedded in paraplast, and horizontal pituitary and cross uterine serial sections (5 μm thickness) were cut. The colchicine-arrested mitotic cells were observed. For the counting of mitotic pituitary cells, sections near the horizontal medial plane were selected. The number of mitotic pituitary cells was counted. In the sections used for the counting, the area of anterior pituitary glands was measured with a planimeter. The mitotic activity was expressed as the number of mitotic cells per mm^2. For the counting of mitotic uterine luminal epithelial cells, sections near the cross medial plane were selected.

Accepted May 25, 1994
Received March 14, 1994